IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

REQUEST FOR FILING NATIONAL PHASE OF PCT APPLICATION UNDER 35 U.S.C. 371 AND 37 CFR 1.494 OR 1.495

To: Hon. Commissioner of Patents
Washington, D.C. 20231

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US)

Atty Dkt: PM / M# /Client Ref.

From: Pillsbury Madison & Sutro LLP, IP Group:

Date: December 20, 2000

This is a **REQUEST** for **FILING** a PCT/USA National Phase Application based on:

1. International Application	2. International Filing Date	3. Earliest Priority Date Claimed
PCT/US99/12825	25 June 1999 (25/06/99)	26 June 1998 (26/06/98)
⇧ country code	Day MONTH Year	Day MONTH Year (use item 2 if no earlier priority)

4. Measured from the earliest priority date in item 3, this PCT/USA National Phase Application Request is being filed within:

(a) ☐ 20 months from above item 3 date (b) ☒ 30 months from above item 3 date,

(c) Therefore, the due date (unextendable) is December 25, 2000

5. Title of Invention METHODS AND COMPOSITIONS FOR MODULATING ANTIGEN-SPECIFIC IMMUNOLOGICAL (HUMORAL) RESPONSES BY TARGETING SUCH ANTIGEN TO APCs IN CONJUNCTION WITH ANTI-CD40 LIGAND ADMINISTRATION

6. Inventor(s) William F. Wade and Douglas Demian

Applicant herewith submits the following under 35 U.S.C. 371 to effect filing:

7. ☒ Please immediately start national examination procedures (35 U.S.C. 371 (f)).

8. ☒ **A copy of the International Application** as filed (35 U.S.C. 371(c)(2)) is transmitted herewith (file if in English but, if in foreign language, file only if not transmitted to PTO by the International Bureau) including:

a. ☒ Request;
b. ☒ Abstract;
c. 42 pgs. Spec. and Claims;
d. 2 sheet(s) Drawing which are ☐ informal ☒ formal of size ☒ A4 ☐ 11"

9. ☒ **A copy of the International Application has been transmitted by the International Bureau.**

10. **A translation of the International Application** into English (35 U.S.C. 371(c)(2))
a. ☐ is transmitted herewith including: (1) ☐ Request; (2) ☐ Abstract;
(3) _____ pgs. Spec. and Claims;
(4) _____ sheet(s) Drawing which are:
☐ informal ☐ formal of size ☐ A4 ☐ 11"
b. ☒ is not required, as the application was filed in English.
c. ☐ is not herewith, but will be filed when required by the forthcoming PTO Missing Requirements Notice per Rule 494(c) if box 4(a) is X'd or Rule 495(c) if box 4(b) is X'd.
d. ☐ Translation verification attached (not required now).

11. ☒ **PLEASE AMEND** the specification before its first line by inserting as a separate paragraph:
a. ☒ --This application is the national phase of international application PCT/US99/12825 filed June 25, 1999 which designated the U.S, and that international application ☒ was ☐ was not published under PCT Article 21(2) in English.--
b. ☒ --This application also claims the benefit of U.S. Provisional Application No. 60/ 090,849 , filed June 26, 1998 .--

RE: USA ~~National~~ Filing of PCT /US99/12825

JC01 Rec'd PCT/PTO 20 DEC 2000
09/720078

12. ☐ Amendments to the claims of the International Application **under PCT Article 19 (35 U.S.C. 371(c)(3)), i.e., before 18th month from first priority date above in item 3, are transmitted herewith (file only if in English) including:**

13. ☒ PCT Article 19 claim amendments (if any) have been transmitted by the International Bureau

14. ☐ Translation of the amendments to the claims **under PCT Article 19 (35 U.S.C. 371(c)(3)), i.e., of claim amendments** made before 18th month, **is attached (required by 20th month from the date in item 3 if box 4(a) above is X'd, or 30th month if box 4(b) is X'd, or else amendments will be considered canceled).**

15. **A declaration of the inventor** (35 U.S.C. 371(c)(4))
a. ☐ is submitted herewith ☐ Original ☐ Facsimile/Copy
b. ☒ is not herewith, but will be filed when required by the forthcoming PTO Missing Requirements Notice per Rule 494(c) if box 4(a) is X'd or Rule 495(c) if box 4(b) is X'd.

16. **An International Search Report (ISR):**
a. Was prepared by ☐ European Patent Office ☐ Japanese Patent Office ☒ Other
b. ☒ has been transmitted by the international Bureau to PTO.
c. ☐ copy herewith (__ pg(s).) ☐ plus Annex of family members (__ pg(s).).

17. **International Preliminary Examination Report** (IPER):
a. ☒ has been transmitted (if this letter is filed after 28 months from date in item 3) in English by the International Bureau with Annexes (if any) in original language.
b. ☐ copy herewith in English.
c.1 ☐ IPER Annex(es) in original language ("Annexes" are amendments made to claims/spec/drawings during Examination) including attached amended:
c.2 ☐ Specification/claim pages #___ claims #
Dwg Sheets #
d. ☐ Translation of Annex(es) to IPER **(required by 30th month due date, or else annexed amendments will be considered canceled).**

18. **Information Disclosure Statement** including:
a. ☐ Attached Form PTO-1449 listing documents
b. ☐ Attached copies of documents listed on Form PTO-1449
c. ☒ A concise explanation of relevance of ISR references is given in the ISR.

19. ☐ **Assignment** document and Cover Sheet for recording are attached. Please mail the recorded assignment document back to the person whose signature, name and address appear at the end of this letter.

20. ☐ Copy of Power to IA agent.

21. ☐ **Drawings** (complete only if 8d or 10a(4) not completed): _ sheet(s) per set: ☐ 1 set informal; ☐ Formal of size ☐ A4 ☐ 11"

22. Small Entity Status ⌀ ☐ is **Not** claimed ☒ is claimed (**pre**-filing confirmation required)

22(a) ___ (No.) Small Entity Statement(s) enclosed (since 9/8/00 Small Entity Statements(s) not essential to make claim)

23. **Priority** is hereby claimed under 35 U.S.C. 119/365 based on the priority claim and the certified copy, both filed in the International Application during the international stage based on the filing in (country) US of:

	Application No.	Filing Date		Application No.	Filing Date
(1)	60/090,849	June 26, 1998	(2)		
(3)			(4)		
(5)			(6)		

a. ☒ See Form PCT/IB/304 sent to US/DO with copy of priority documents. If copy has not been received, please proceed promptly to obtain same from the IB.
b. ☐ Copy of Form PCT/IB/304 attached.

24. Attached:

RE: USA National Filing of PCT/US99/12825

09/720078
JC01 Rec'd PCT/PTO 20 DEC 2000

25. **Preliminary Amendment:**

25.5 Per Item 17.c2, **cancel original** pages #___, claims #___, Drawing Sheets #

26. **Calculation of the U.S. National Fee (35 U.S.C. 371 (c)(1)) and other fees is as follows:**
Based on amended claim(s) per above item(s) ☐ 12, ☐ 14, ☐ 17, ☐ 25, ☐ 25.5 (hilite)

Total Effective Claims	31	minus 20 =	11	x $18/$9 =	$99	966/967
Independent Claims	4	minus 3 =	1	x $80/$40 =	$40	964/965
If any proper (ignore improper) Multiple Dependent claim is present,				add$270/$135	+0	968/969

BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(4)): →→ **BASIC FEE REQUIRED, NOW** →→→→

A. If country code letters in item 1 are **not** "US","BR","BB","TT","MX","IL" "NZ", "IN" or "ZA"

See item 16 re:

1.	Search Report was not prepared by EPO or JPO - - - - - - - - -	add$1000/$500	960/961
2.	Search Report was prepared by EPO or JPO - - - - - - - - - - -	add$860/$430 +500	970/971

SKIP B, C, D AND E UNLESS country code letters in item 1 are "US","BR","BB","TT","MX","IL", "NZ", "IN" or "ZA"

→ ☐	B.	If USPTO did not issue both International Search Report (ISR) and (if box 4(b) above is X'd) the International Examination Report (IPER), - - - - - - - - - - - - - - - - - - -	add$970/$485 +0	960/961
☐	C.	If USPTO issued ISR but not IPER (or box 4(a) above is X'd), -	add$710/$355 +0	958/959
→ ☒	D.	If USPTO issued IPER but IPER Sec. V boxes not all 3 YES, -	add$690/$345 +345	956/957
→ ☐	E.	If international preliminary examination fee was paid to USPTO and Rules 492(a)(4) and 496(b) satisfied (IPER Sec. V all 3 boxes YES for all claims), - - - - - - - - - - - - -	add $100/$50 +0	962/963
			SUBTOTAL = $984	

28. If Assignment box 19 above is X'd, add Assignment Recording fee of ----$40 +0 (581)

29. Attached is a check to cover the - **TOTAL FEES** $984

Our Deposit Account No. 03-3975
Our Order No. _____ C# | _____ M#

CHARGE STATEMENT: The Commissioner is hereby authorized to charge any fee specifically authorized hereafter, or any missing or insufficient fee(s) filed, or asserted to be filed, or which should have been filed herewith or concerning any paper filed hereafter, and which may be required under Rules 16-18 and 492 (missing or insufficient fee only) now or hereafter relative to this application and the resulting Official document under Rule 20, or credit any overpayment, to our Account/Order Nos. shown above for which purpose a duplicate copy of this sheet is attached.
This CHARGE STATEMENT does not authorize charge of the issue fee until/unless an issue fee transmittal form is filed

Pillsbury Madison & Sutro LLP
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By Atty: Robin L. Teskin **Reg. No.** 35,030
Sig: _____ **Fax:** (202) 822-0944
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NOTE: File in duplicate with 2 postcard receipts (PAT-103) & attachments.

2/PRTS

09/720078

PCT/US99/12825

JC01 Rec'd PCT/PTO 20 DEC 2000

METHODS AND COMPOSITIONS FOR MODULATING ANTIGEN-SPECIFIC IMMUNOLOGICAL (HUMORAL) RESPONSES BY TARGETING SUCH ANTIGEN TO APCs IN CONJUNCTION WITH ANTI-CD40 LIGAND ADMINISTRATION

This invention was supported by National Institutes of Health Grant Number AG14782.

Field of the Invention

The present invention relates to methods, immunoconjugates, and compositions containing for enhancing the immune response to an antigen, e.g., a viral, bacterial, or tumor antigen, by targeting such antigen to a class II molecule on an antigen presenting cell (APC), optionally in conjunction with an anti-CD40 ligand. These methods, immunoconjugates, and compositions have particular application in boosting the humoral immune responses of aged or immuno-compromised individuals who otherwise would elicit an inadequate immune response to the particular antigen, e.g., a viral, bacterial, or tumor antigen.

Background of the Invention

The administration of antigen to a particular target, e.g., a tumor cell, parasite, virus or bacterial cell, in order to elicit a specific immune response against such target antigen is well known in the art. Such immune response will be characterized by the production of antibodies specific to the target antigen by the hosts B-cells. Ideally, the immune response to the target antigen will be efficacious, i.e., will elicit a prophylactic or therapeutic response to a particular target, e.g., a disease causing agent. For example, a prophylactic or therapeutic response may be elicited against a tumor cell, pathogen, virus, bacterial, or another infectious agent.

While this strategy is effective in some instances, e.g., in the context of bacterial vaccines, it is not always effective. For example, some antigens even when administered at high or repeated dosages may not elicit a sufficient immune response to be therapeutically or prophylactically effective. This may occur, e.g., when the target antigen is one expressed at low levels on the target cell, or if the target antigen is an autoantigen or other antigen having a conserved structure in different mammals making the production of antibodies difficult or even impossible by conventional methods.

One group of individuals wherein elicitation of effective immune responses to target antigens is particularly problematic is aged individuals. On average, the aged immune system is less effective at responding to immunogens in relation to the immune system of younger individuals. For example, it is known that aged individuals exhibit a higher frequency of defects involving T-cell activation, antibody production, and antigen presentation.

It has been observed in both mice and humans that the aging process has a negative impact on the immune system which results in a reduced ability to generate high affinity antibodies or to achieve long-lasting immune response after vaccination. (Miller, R.A., *Science*, 283:70-74 (1996).)

While the explanation for the reduced efficacy of the aged immune system is unresolved, it appears to involve the impairment of different immune functions, including various T-cell functions. Based thereon, T-cells from aged individuals have been extensively studied in an effort to explain and obviate impairments in T-cell activation associated with aging. The results of these studies have indicated that T-cell proliferation, generation of CTL effectors, and the delivery of T-cell helpers for B-cell induction is reduced in aged individuals. (Miller, R.A. (Id.).) It has been hypothesized that the reduced efficacy of T-cells in aged individuals to elicit an effective immune response may be attributable to

the reduced production of IL-2. (Thomas et al, *J. Immunol.*, 127:2101-2106 (1981).)

The lack of effective T-cell help may have a negative impact on B-cell activation in aged individuals, resulting in an impairment of effective humoral immune responses. Moreover, it has also been reported that the B-cells of aged individuals may have intrinsic defects. For example, it has been reported that the selection of V families for specific antigen in old mice may be different than in young mice. (Riley et al., *J. Immunol.*, 143:3798-3805 (1989); Nicoletti et al., *J. Immunol.*, 150:543-549 (1993).) Also, it has been reported that the antibody response to some protein antigens, e.g., Flu antigens is reduced in aged mice. (Effros et al., *J. Genrontol*, 46:B142-47 (1991).)

A particularly debilitating effect that has been observed in the B-cell compartment of aged mice is the lack of Ig locus hypermutation that normally occurs in the germinal centers (of normal or younger mice). (Zheng et al., *Immunol. Rev.*, 160:63-77 (1997).) It has been observed that germinal centers of spleens of aged mice are markedly reduced in relation to younger mice following immunization. (Zheng et al. (*Id.*).) Consequently, the generation of high affinity antibodies and B-cell expansion is significantly diminished in aged individuals. (LeMaoult et al., *Immunol. Rev.*, 160:115-126 (1997).)

Notwithstanding the many adverse age-associated changes that occur in the B and T-cell compartments of aged immuno systems, some evidence suggests that the professional antigen presenting cell compartment of aged individuals apparently remains largely intact. (Miller, R.A., *Science*, 273:70-74 (1996).) However, some reports suggest that the efficacy of specific subsets of antigen presenting cells (APCs) may be affected by the aging process. For example, two studies suggest that APCs from aged individuals are less efficient at stimulating immunity than APCs of younger individuals. (Maletto et al., *Mech. Aging*

Devel., 88:39-47 (1996); Garg et al., *Infect. Imm.*, 64:4456-4462 (1996).) Also, it has been reported that the number of Langerhan cells is 40% lower in the skin of aged relative to younger mice. (Belsito et al., *J. Immunol.*, 143:1530-1536 (1989).)

A key cell for elicitation of effective immune responses is the dendritic cell. (Pierre et al., *Nature*, 388:778-782 (1997).) T-cell priming is linked to DC biology with respect to DC maturation responses to inflammatory stimuli. (Pierre et al., (*Id.*); Cella et al., *Nature*, 388:782-787 (1997).) Accordingly, it has been hypothesized that age-associated immune deficiencies may involve dendritic cell impairments.

Because of the afore-discussed age-associated impairments to the immune system, aged individuals, on average, have a greater probability of having a compromised immune system which may make eliciting an effective immune response to a target antigen difficult or even impossible by conventional immunization protocols. Conventional methods for enhancing immune responses to target antigen include, by way of example, administering such antigen in conjunction with an adjuvant such as Saponin or Alum, conjugating such antigen to a carrier protein, multimerization (so as to present multiple copies of the particular antigen to the host's immune system), increased antigen dosages, repeated antigen administration, and the use of specific delivery systems, e.g., liposomal delivery systems. While these methods are often effective in persons with normal immune systems, they may be largely ineffective in persons with impaired immune systems, e.g., aged individuals. Also, such methods may be ineffective in individuals with immune impairments that are the result of disease, drug therapy, or genetic defects.

Therefore, it would be beneficial if methods for boosting immune responses to target antigens, e.g., viral, bacterial, or tumor antigens, in

individuals in need of such treatment could be identified. These methods will be advantageous for any subject in need of such treatment, but especially would be beneficial in subjects that are immuno-compromised or immuno-impaired, such as aged individuals.

Brief Description of the Invention

Toward that end, the present inventors have surprisingly discovered that the immune response to a target antigen, especially the humoral immune response in an aged immune system, or DTH type T-cell immune response, can be enhanced by targeting such antigen to surface molecules on APCs, e.g., class II molecules or professional APCs, optionally in conjunction with an anti-CD40 ligand.

While it has been previously reported that the humoral immune responses to a target antigen may be boosted in young individuals by targeting such antigen to APCs (Snider et al., *J. Exp. Med.*, 171:1957-1963 (1990); Estrada et al., *Vaccine*, 13:901-907 (1995); and Snider et al., *Immunol.*, 90:323-329 (1997), it could not have been reasonably predicted that such methodology would have any significant benefit in aged individuals (given their compromised immune systems, including APCs, relative to young individuals).

As discussed in detail *infra*, the present inventors examined the efficacy of targeting an antigen to class II molecules on professional APCs from aged individuals as a means of boosting the immune response to such antigen. Also, it was examined what other molecules expressed as APCs might be exploited for antigen targeting in order to boost immune responses to such antigen, in particular CD38 (expressed on primary follicular B-cells and mantle B-cells); CD40 (expressed on DCs, Mø and B-cells); and CD11c (expressed on DCs and myeloid cells). The results of these experiments indicate that while class II targeting was very effective at stimulating primary and secondary humoral

immune responses in aged individuals, CD40 and CD38 were not. By contrast, CD11c was partially effective, i.e., it induced a primary humoral response (observed when challenge was given IV +6 days post-immunization rather than Sub Q +35 days). These results suggest that while APC surface molecules in general can signal or access the endocytic pathway, they do not function equivalently as effective substrates for antibody-targeted antigen, and for enhancing humoral immune responses.

Also, it was surprisingly discovered that the immune response to a target antigen can in some instances be synergistically enhanced by targeting such antigen to specific APC surface molecules, e.g., class II molecules or CD11c, in combination with a CD40 ligand, e.g., by including in the inoculum containing the targeting antibody an anti-CD40 antibody. However, whether the anti-CD40 antibody has a beneficial effect on humoral immunity appears to depend upon the specifics of the particular antigen system. The results obtained, discussed *infra*, suggest that the inclusion of anti-CD40 in the targeting antibody containing inoculum can enhance or suppress the humoral immune response, and that this is apparently antigen-dependent.

Therefore, the present invention is based on several surprising discoveries, namely (i) that humoral immune responses and Th1 type response (DTH response) to desired antigens can be enhanced in aged and other similarly immuno-compromised individuals by targeting such antigen to specific APC molecules, e.g., class II molecules and CD11c; and (ii) that humoral immune responses to some target antigens can be synergistically enhanced or suppressed by targeting such antigen to specific APC molecules, e.g., class II molecules or CD11c, and further administering an anti-CD40 ligand, e.g., an anti-CD40 antibody.

Objects of the Invention

It is an object of the invention to enhance humoral immune responses to target antigens, especially in aged and other immuno-compromised individuals.

It is a more specific object of the invention to enhance the humoral immune response to a target antigen in an aged or immuno-compromised individual *in vitro* or *in vivo* by targeting such antigen to specific APC molecules, e.g., class II molecules or CD11c.

It is another specific object of the invention to synergistically enhance the humoral immune response or Th1 immune response (DTH response) to a target antigen, e.g., RSV F protein, by targeting such antigen to specific APC molecules, e.g., class II molecules or CD11c, and additionally administering a CD40 ligand, e.g., an anti-CD40 antibody.

It is still another object of the invention to suppress the immune response to a desired antigen by targeting such antigen to specific APC molecules, e.g., Class II molecules or CDllc, and further administering an anti-CD40 ligand, e.g., anti-CD40 antibody.

Detailed Description of the Invention

The present invention is its broadest embodiments relates to methods and compositions for boosting humoral immune responses, e.g., primary and secondary humoral response, to a target antigen, e.g., RSV fusion protein, by targeting such antigen to specific APC molecules, e.g., class II molecules or CD11c. In the preferred embodiment, this will be effected in aged individuals or other individuals having compromised immune systems.

This will preferably be effected by attaching a desired antigen, e.g., a viral antigen or tumor antigen, to a moiety that targets such antigen to a specific molecule expressed on the surface of an antigen presenting cells. Preferably, such molecule will comprise a class II molecule or CD11c. However, it is

anticipated that other molecules expressed on the surface of APCs may be identified that have a similar beneficial effect on antigen targeting and humoral immunity.

Preferably, the moiety that targets the antigen to the APC molecule will be an antibody or antibody fragment that specifically binds such molecule. Most preferably, the targeting moiety will comprise a monoclonal antibody that specifically binds to a class II molecule, i.e., an anti-MHC class II monoclonal antibody or fragment thereof. Less preferably, the targeting moiety will comprise a monoclonal antibody that specifically binds CD11c or a fragment thereof.

Antibodies to MHC class II molecules, i.e., anti-HLA and anti-IA antibodies are well known with many being commercially available. Alternatively, monoclonal antibodies to specific MHC class II molecules can be produced by known methods for manufacture of monoclonal antibodies. Antibodies specific to MHC class II molecules are disclosed, e.g., in Flajnik et al, *Mol. Immunol.* 27(5):4541-4562 (1990); Lanzavecchia et al, *Eur. J. Immunol.*, 17:105-111 (1987); and Dutia et al, *Immunol.* 70:27-32 (1990). Also, such antibodies are available from the American Type Culture Collection, Rockville, Maryland.

As the focus of the present invention is the treatment of human subjects, and most preferably aged or otherwise immuno-comprised subjects, monoclonal antibodies or fragments that specifically bind human MHC class II molecules or other molecules expressed on human APCs will be attached directly or indirectly to desired antigens.

Other molecules on APCs that may be useful for targeting antigen can be identified by one skilled in the art based on the teaching of this application and knowledge in the art relating to such molecules. Other such molecules include,

by way of example, CD38, dendritic cell antigens, follicular dendritic cell antigens, and Fc molecules.

The target antigen can be any antigen to which an antigen-specific immune response is therapeutically or prophylactically beneficial, e.g., an antigen expressed by a tumor cell or disease causing organism such as a virus, bacteria, or other pathogen. The antigen can be a purified or recombinant protein or peptide or a whole organism or cell such as an intact viron or tumor cell. Preferred examples of target antigens include tetanus and diphtheria toxins, flu antigens, RSV antigens such as RSV F protein, polio antigens, HIV gp120 and other HIV antigens, and breast, ovarian or prostate tumor antigens.

Preferably, the targeting antibody will be a monoclonal antibody or a fragment thereof that binds to an antigen which is expressed by an antigen-presenting cell (APC). Suitable antibodies and fragments include, by way of example, human monoclonal antibodies, chimeric antibodies (antibodies which contain human constant domains and non-human variable regions), humanized antibodies, bi- specific antibodies, and fragments thereof that bind antigen e.g., Fv, Fab, and $F(ab)'_2$ fragments. Most preferably, the antibody will be a chimeric humanized monoclonal antibody or a human monoclonal antibody, so as to avoid or minimize elicitation of an immunological response against the antibody.

Covalent attachment of the antibody to the antigen may be effected by known methods for effecting the conjugation of proteins. Moreover, methods for attaching desired moieties to antibodies are well known in the art. Typically, such methods will effect attachment at available functional groups on antibody molecules, which include carboxyl and amino moieties comprised in the amino acids contained therein, disulfide or thiol linkage by attachment to cysteine residues, and attachment to carbohydrate moieties comprised in the Fc region, via Schiff's type linkages. Also, bifunctional linkers may be utilized to facilitate

attachment of the antibody to the antigen. Alternatively, the antigen and antibody may be expressed as a fusion protein in a suitable host cell, i.e., by expressing the DNA encoding such antigen which is fused to an antibody which encodes an antibody that binds to an antigen expressed by a cell involved in APC function, for example, an MHC class II antigen, thereby avoiding the need to attach these moieties by chemical means. Suitable systems for expressing recombinant antibodies, and fusions thereof, are well known and are commercially available.

The antigen which is directly or indirectly fused to the antibody will typically comprise an antigen which is specific to an etiological agent, for example, an antigen expressed by or a product of a tumor cell, a virus, a bacterium, a parasite, or other infectious agent. Examples thereof include, for example, purified recombinant proteins, peptides, whole organisms such as intact virons, flu, polio, tetanus and diphtheria toxins, TB antigens, and HIV gp120 antigens, as well as tumor antigens such as breast, ovarian and prostate tumor antigens. Specific examples thereof include, by way of example, antigens expressed by HIV such as gp160, Gag, Pol, New, The, and Reb; malarial antigens such as the CS protein and sporozooyte surface protein 2; hepatitis B surface antigens such as pre-S1, pre-S2, HBc Ag, and HBe Ag; influenza antigens such as HA, NP and NA; hepatitis A surface antigens; hepatitis C surface antigens; herpes virus antigens, such as EBV GP340, EBV GP85, HSV gB, HSV gD, HSV gH, HSV early protein product, cytomegalovirus gB, cytomegalovirus gH and IE protein gp72, respiratorial syncytial viral antigens such as the F protein, G protein and N protein; leprosy antigens, listeriosis antigens, tumor antigens such as carcinoma CA, carcinoma mutated EGF receptor, prostate carcinoma specific antigen (PSA), prostate specific membrane associated antigen, carcinoma associated mucin, carcinoma p21, carcinoma p53,

melanoma MPG, melanoma p97, MAGE-1, MAGE 3, gp100, MART 1, melanoma antigen gp75, carcinoma NEU oncogene product, and ras protein. Other examples include papillomavirus antigens such as the L1 and L2 proteins., and Lyme's disease antigens. Typically the antigen will be one expressed on the surface of a particular target, for example, a tumor cell, a pathogen, a bacterium or a virally infected cell.

As discussed above, and demonstrated by the results in the examples, it has further been discovered that the humoral immune response to a desired antigen can be synergistically enhanced, or alternatively suppressed dependent on the antigen system, by both targeting such antigen to a desired APC molecule, e.g., MHC class II molecule, *in vitro* or *in vivo*, and further administering a CD40 ligand, e.g., an antibody to human CD40 or a fragment thereof.

Based on the results in the Examples, which follow, it has been surprisingly found that the further administration of an anti-CD40 antibody may synergistically enhance or suppress the humoral response to the antigen which has been targeted to a molecule expressed by an APC, e.g., class II MHC molecule. Whether the immune response is enhanced or suppressed is apparently antigen-dependent as, dependent on the particular antigen system, the inclusion of the anti-CD40 ligand may enhance, have no effect, or elicit an adverse effect on the humoral immune response to the target antigen. One of skill in the art can ascertain by routine experimentation whether the anti-CD40 ligand enhances or suppresses humoral immune response to a target antigen.

The anti-CD40 ligand will similarly preferably be a monoclonal antibody or fragment thereof, and most preferably will be a humanized antibody, human monoclonal antibody or a fragment thereof that binds human CD40. As noted, the antigen-antibody complex of (i) and the anti-CD40 antibody of (ii) will preferably be administered in combination, or substantially contemporaneously,

so as to elicit the desired immunological response, i.e., enhanced or suppressed humoral immune response to the particular antigen. This will most typically be effected by administering a vaccine composition which comprises the antigen-antibody complex, e.g., an antigen-anti-MHC class II monoclonal antibody complex and an anti-CD40 antibody, wherein the amount thereof is sufficient to elicit the desired immunological response to the target antigen. However, alternatively, the antigen-antibody complex and the anti-CD40 antibody may be administered separately, provided that they are administered substantially contemporaneously. By "substantially contemporaneously", applicants mean that the antigen-antibody complex and the anti-CD40 antibody are administered sufficiently proximate in time to elicit the desired synergistic effect on humoral immunity, i.e., typically within about twelve hours of one another, more preferably within one to four hours, and most preferably, at the same time. If administered separately, these antibodies may be administered in either order, provided that the antigen-antibody complex and the anti-CD40 antibody are administered substantially contemporaneously. The antigen-antibody complex and the anti-CD40 antibody may be combined with known carriers used in vaccine compositions which include, by way of example, sterile water, buffered saline, known adjuvants, such as Alum and Saponin, and other pharmaceutically acceptable carriers and diluents known to be suitable for use in vaccine formulations.

The route of administration of the subject antigen-antibody complex and anti-CD40 antibody may be oral, parenteral, by inhalation, or topical. The term parenteral as used herein includes, by way of example, intranasal, intraperitoneal, intravenous, intramuscular, subcutaneous, rectal, vaginal, and intratumoral administration. Subcutaneous and intramuscular forms of parenteral administration are generally preferred. The effective dosage of the antigen- anti-

MCH molecule antibody conjugate and the anti-CD40 antibody, will comprise dosages that are sufficient in order to elicit the desired immunological, i.e., humoral immune response to the target antigen. Typically, an effective dosage of each will range from about 0.005 to 100 milligrams per kilogram body weight per day. However, the exact dosage will vary dependent upon factors such as the particular target antigen, its relative antigenicity, the level of expression of such antigen by the particular etiological agent, for example, a tumor cell or virus, and the condition of the host treated.

Also, the compositions used in the claimed methods may further comprise other materials known to effect immunological responses and in particular humoral immune responses. Examples thereof include, by way of example, adjuvants such as Alum and saponin, immune stimulating peptides such as muramyl tripeptide and derivatives thereof, and other moieties known to enhance immunological responses. One particular adjuvant which may be useful comprises the adjuvant disclosed in U.S. Patent No. 5,585,103, which comprises a microfluidized formulation which includes a stabilizing detergent such as a Tween type detergent, a micelle-forming agent which results in the formation of a micelle-like structure such as polymers and surfactants including pluronic and tetronic type surfactants and a biologically acceptable oil such as Squalene, Squalene, Eicosane, Tetratetracontane, glycerol, peanut oil and other vegetable oils. The patent discloses that these adjuvants are advantageous in that they induce a cytotoxic T lymphocyte response to the particular antigen. The incorporation of such adjuvants in the subject compositions may further enhance the immunological response related to the target antigen. However, it should be noted that some of such materials potentially could denature the antibody. The effects of such agents, in particular whether they result in any enhancement or

suppression of the immunological response an be determined by one skilled in the art.

As discussed above, the subject methods and compositions are advantageous, in that they can be used to potentiate or suppress the immune response, in particular the humoral immune response to a particular antigen, for example, an autoantigen, allergan, tumor antigen, bacterial antigen, antigen expressed by parasite, etc. The mechanism whereby the subject methods work has not been clearly established to date. However, it may involve enhancement or suppression of antigen-presenting cell (APC) function, and B and T-lymphocyte function. This will be confirmed based on additional experiments which will assess the effects of the subject methods on APC function and B and T-cell function in germinal centers. The subject methods also may enhance humoral immune responses by a mechanism which involves at least one of the following: (i) stabilization of MHC class II antigen complexes, (ii) enhanced expression of APC costimulatory molecules, (iii) potentiation and enhancement of development of germinal center B-cells, (iv) improved expansion and activation of antigen-specific T-cells, and (v) increased antigen on APC in the context of stimulatory signals, i.e., targeting low doses. (For maximal effect.)

The subject methods are applicable especially in therapeutic contexts wherein an enhanced immunological response, and in particular an enhanced humoral immune response to a desired antigen, is therapeutically or prophylactically beneficial. This includes, in particular, the production of therapeutic and prophylactic vaccines to infectious organisms such as viruses, bacteria and other pathogens, and anti-tumor vaccines. The subject methods should result in improved efficacy, without the concomitant risk associated with other adjuvants. For example, some adjuvants, such as Freund's complete adjuvant, are associated with adverse side effects such as granulomas. The

subject methods and materials should not elicit any adverse effects on the immune system.

The subject methods are useful for enhancing immunological responses to different antigens. Suitable antigens are discussed above, and include, in particular, antigens expressed by cancer cells, viruses, bacteria, pathogens and other disease targets. The present method should particularly be suitable in the context of producing antibodies to antigens which do not elicit significant humoral immune responses, in particular, tumor antigens, and other antigens, the structure of which is conserved in different species. Particularly contemplated applications of the subject methods include tetanus and diphtheria toxins, flu, polio and HIV gp120 and gp160 antigens, RSV antigens (e.g., fusion protein), and breast, ovarian and prostate tumor antigens. These are particularly contemplated because suitable targets, i.e., antigens are known and available.

As discussed, a significant application of the present invention is for boosting humoral immune responses in individuals having impaired immune systems, e.g., because of age, genetic defects, or disease. In particular, the results of the experimental examples suggest that the humoral immune response can be potentiated in "aged" individuals.

In the context of the invention, "aged" will refer to the average age at which the immune system of an individual, preferably human, becomes, on average, impaired relative to the immune system of a juvenile. For the purposes of this application, "aged" humans will typically refer to individuals who are at least 50 years of age, more typically 60 years or older.

Also, immuno-compromised individuals who are amenable to treatment according to the invention include AIDS subjects, transplant recipients who are on immunosuppressants, and individuals with other diseases or genetic defects that result in compromised immune systems.

The efficacy of the subject invention has been confirmed in mice, which were treated according to the invention. In particular, it was demonstrated that mice administered an antigen-anti MHC class II monoclonal antibody and an anti-CD40 monoclonal antibody, in both young or old mice, resulted in significantly enhanced yields of IgG antibody to the antigen, which is normally associated with high affinity, protective immune responses. Such antibody responses were significantly enhanced in relation to mice which were administered the antigen alone.

As discussed above, the antigen-antibody conjugate and the anti-CD40 antibody will preferably be comprised in the same formulation. However, alternatively they may be comprised in separate compositions, which are administered simultaneously or substantially contemporaneously. Advantageously, such antigen-antibody complex and anti-CD40 may be formulated in the form of a kit, wherein such moieties may be packaged separately or in combination. Such kits will include conventional carriers, stabilizing moieties, and other ingredients conventionally included in vaccine compositions including, by way of example, surfactants, bacteriocides, pharmaceutically acceptable oils, preservatives, fungicidal agents, other adjuvants, *et seq*.

While a significant application of the invention involves enhancing humoral immune responses, and specifically the generation of high affinity antibodies and long-lasting antigen-specific immune responses, the subject methods alternatively may be used to suppress immune responses to particular antigens. As discussed above, it has been observed that for some antigen systems targeting of antigen to APCs (e.g., by attachment of antigen to Class II antibody) and administration of anti-CD40 ligand (anti-CD40 antibody) may suppress antigen-specific immune responses. While the actual mechanism by

which this occurs is unclear, it may involve effects on the germinal center. In particular, it is hypothesized that the anti-CD40 ligand may be causing cells contained therein, e.g., T- and B-cells, to be in a state of anergy, i.e., quiescence, resulting in reduced proliferation. This may result in a suppression of antigen-specific T- and B-cell functions, and thereby resulting in a suppression of the host immune response to the particular antigen. This aspect of the invention is beneficial in contexts wherein immune responses are unwanted, e.g., gene therapy, transplantation, autoimmune disease, and allergic reactions. In particular, the subject method may be used to suppress immune responses to autoantigens, administered therapeutic agents such as antibodies, transplantation antigens (xeno and alloantigens), vectors and cells used for cell and gene therapy, and other antigens wherein suppression of an antigen-specific immune response is therapeutically beneficial.

As discussed, whether the anti-CD40 antibody suppresses or potentiates antigen-specific immune responses is apparently a function of the antigen system. It is anticipated, based on the information in this application, that those skilled in the art will be able to design suitable immunization protocols for suppressing or enhancing an antigen-specific immune responses by targeting such antigen to an APC molecule, e.g., Class II molecule, together with the administration of anti-CD40 antibody.

In order to further describe the invention, the following examples are provided. These examples are illustrative only and should not be construed to limit the scope of the invention.

EXAMPLES

The Materials and Methods used in the examples is set forth below.

Materials and Methods:

Conjugate and Targeting Construct Preparation

Hen egg lysozyme/anti-class II conjugates were produced as described by Snider et al. (Snider et al (Id.).) Briefly, HEL (Sigma Chemical Co. St. Louis MO) and protein A, purified anti I-A^k mAb (10.2.16) were substituted with the heterobifunctional cross-linking reagent N-succinimidyl 3-(2-pyridydithio) propionate (Pierce Chemicals, Rockford, IL) and reacted to form heteroconjugates. The HEL/anti-class II complexes were separated from free HEL by Sephadex G75 chromatography to yield a class II targeting construct with a final antigen concentration of 320 µg/mL. Avidin/mAb conjugates were produced by biotinylating 500 µg of purified mAbs: anti-class II (10.2.16), anti-CD38, anti-CD40 (FGK 115) or anti-CD11c with 55 µg biotin (Pierce Chemicals, Rockford IL). The 10.2.16 (Ui et al, *Curr. Topics Microbiol Immunol.*, and 81:115-129 (1990), FGK-115 mAb (a gift from Dr. G. Roinik Basal Switzerland) were produced in-house by protein-A affinity chromatography Biotinylated mAbs were dialyzed against PBS (pH 7.4) overnight at 4°C before conjugation with 652.6 µg avidin (Sigma Chemical Co., St. Louis MO). Antigen/mAb conjugates and titrated amounts of free HEL or avidin were separated on 14% SDS-PAGE gels under reducing and non-reducing conditions to determine the amount of bound and unbound antigen in conjugate preparation.

Immunizing Reagents

Immunizing inoculum for all experiments was prepared in sterile PBS (pH 7.4) in a final volume of 100 µl and then sterile filtered (0.2 µm syringe filters, Millipore, Bedford, MA) before subcutaneous or intraperitoneal inoculations. For *in vitro* antigen loading of DC, a spleen was removed from either an aged or young mouse that was treated for 10 days with human recombinant Flt-3 Ligand

[Pulendran et al, E. Developmental pathways of dendritic cells *in vivo*: distinct function, phenotype and localization of dendritic cell subsets in FLT3 ligand-treated mice, *J. Immunol.*, 159: 2222-2231 (1997) Immunex Corp., Seattle, WA.] Single cell suspensions were made by passage through sterile nylon dialysis mesh; red blood cells were lysed with ammonium chloride and cell suspensions were T-cell-depleted using a cocktail of anti-CD4 mAb, anti-Thy1.1 mAb plus rabbit complement (Low-Tox; Accurate Chemical Co., Westbury, NY).

B-cell depletion was effected by anti-IgM panning for 60 minutes at 37°C (Zymed Laboratories, Inc., So San Francisco, CA). (Macrophages stick to the panning plates, however, Flt-3 treated DC do not (W. F. Wade, personal observation).) In our hands the purity of DC obtained from young and old mice by this method was about 45%, as evidenced by CD11c staining (data not shown). HEL/anti-class II conjugate was added to cells resuspended in ~300 μl volume, incubated at 4°C for 30 minutes and washed twice with PBS (pH 7.4). Fifty million cells were plated at 10^6/mL, incubated at 37°C in 5% CO_2 for four hours prior to the addition of 10 μg/ml anti-CD40 mAb or 3.2 μg soluble HEL and incubated overnight. Cells were scrapped, washed one time in PBS, counted and inoculated into age-matched mice subcutaneously along the right flank.

Animal Manipulation

Young (8-12 weeks) and old (15-19 months) female CBA/jNIA mice were purchased from the National Institute of Aging mouse colony. They were housed in the Animal Resources Center at Dartmouth Medical School where they were provided with TekLad rodent diet (Harlan, Madison, WI) and water *ad libitum*. Mice were anesthetized using Metophane (Mallinckrodt Veterinary, Inc., Mundelein, IL) and inoculated with sterile solutions using tuberculin syringes fitted with a 27 ga. needle on the right flank for subcutaneous inoculations or the right side of ventral centerline for intraperitoneal inoculations.

Blood collection at different time points in the experiments was performed on anesthetized mice through the retro-orbital sinus. Immunizing inoculations were performed on day 0 followed by serum collection to assess primary responses at +21 days. Mice were challenged subcutaneously with either 25 μg of soluble HEL or avidin 14 days later (+35d) and bled for secondary response antisera on +45 days. In one experiment the mice were challenged intravenously with 25 μg of soluble avidin.

Elisa Assays

96 well Elisa/RIA plates (Costar, Corning, NY) were coated with 10 μg/mL HEL or Avidin in PBS/0.04% sodium azide for four hours and then washed and stored at 4°C until used. Serial two-fold dilutions of experimental sera (prebleed, primary or secondary antisera) or hyperimmune positive control sera were incubated in coated plates overnight at 4°C in blocking buffer (BBS; 0.05% Tween 20; 1mM EDTA; 0.25% BSA; 0.04% sodium azide) at initial dilutions of between 1:100-200 or 1:2,000. Horseradish peroxidase, goat, anti-mouse IgG, IgM, IgA, or IgG2a (KPL, Gaithersburg, MD) were incubated at dilutions of 1:1,000 in BB (BBS without sodium azide) for 2 hours at room temperature followed by extensive washing. The color reaction was developed using ABTS (KPL, Gaithersburg, MD) for 30 minutes and quantified using a Molecular Device (Menlo Park, CA) microplate reader set for 405 nm. Serum titers are reported as the reciprocal of the last dilution that was above zero after subtracting twice the mean optical density values for blank background wells. Prebleed sera was negative at the level of the background wells.

The invention will now be described in more detail in the following Examples.

EXAMPLE 1

Anti-Class II-Targeted Antigen Constructs Can Improve the Humoral Response of Old Mice

One of the goals in this study was to investigate the efficacy of class II-targeted antigen at inducing immune responses in old mice. Female CBA/jNIA mice that were 18-19 months of age, or 10-12 weeks of age, were immunized subcutaneously with (i) HEL/alum, (ii) and (iii) HEL conjugated to anti-I-A^k mAb (10.2.16) with or without accompanying anti-CD40 mAb (rat, FGK-115), (iv) HEL/alum plus anti-CD40 or (v) HEL/alum plus control rat IgG. Following this initial immunization, mice were boosted at the site of the initial immunization 34 days later with 25 μg of HEL in PBS and then bled 11 days after the booster. ELISA results contained in Table 1 below demonstrated that HEL in alum is a marginal antigen for both young and old mice (Table 1).

TABLE 1

Anti-Class II mAb targets HEL for effective humoral immune responses.

Group[1]	Old				Young			
HEL/Alum	100[2]	100	100	**100[4] (0)**	100	400	400	**300 (100)**
HEL/anti-class II	800	200	800	**600 (200)**	1,600	3,200	400	**1,733 (811)**
HEL/anti-class II + anti CD40	3,200	1,600	1,600	**2,133 (533)**	6,400	6,400	6,400	**6,400 (0)**
HEL/Alum+ anti CD40	<100	<100	<100		100	100	100	**100 (0)**
HEL/Rat IgG	<100[3]	<100	<100		<100	<100	<100	

[1] CBA/jNIA female mice, 10-12 week old mice or 18-19 month old mice were immunized subcutaneously on day 1 with HEL/alum (10μg) or 3.5 μg of HEL

conjugated to 10.2.16 (anti-class IimAb) or the anti-class II mAb conjugate and 13.5 μg of anti-CD40 mAb, Hel/alum (10μg) plus 10μg anti-CD40 or HEL/alum (10μg) and 10μg of rat IgG.

[2] Mice were challenged on day +34, subsequently with 25 μg of soluble HEL and then bleed 11 days later. Anti-HEL serum Ig titers were determined for individual mice by ELISA. Prebleed ELISA titers were all 100.

[3] An ELISA titer of 100 is negative. Sample titers were determined by subtracting the sample's absorbance from twice the mean of the blank control wells.

[4] Geometric mean and standard error of mean.

Targeting HEL with anti-class II mAb improves the response in both groups compared to HEL in alum immunization. As for other protein antigens, young mice respond better to HEL than older mice. The anti-HEL serum Ig response induced by class II-targeted HEL can be augmented if anti-CD40 mAb is included in the inoculum. Using HEL/alum with anti-CD40 mAb or with control rat IgG did not significantly enhance the humoral response to HEL. Additional ELISAs were performed to determine that the subclass of anti-HEL serum Ig was IgG1 with little IgM, IgA, and IgG2a being detected (data not shown).

A second experiment, with a newly generated targeting construct, was conducted to determine if there was a particular route of administration for the targeted HEL that was preferred. In addition, we investigated the affect of increased amounts of anti-CD40 mAb at the time of the primary inoculation. These results are in Table 2 below.

TABLE 2

Anti-class II targeted antigen delivered subcutaneously or intraperitoneally can promote a humoral response to HEL.

Young Mice[1]	21 day			
Intraperitoneal HEL/anti-class II[2]	128k[4]	64k	32k	**74.6k[6] (28k)**
HEL/anti-class II + 10 μg anti-CD40	64k	64k	64k	**64k (0)**
HEL/anti-class II + 100 μg anti-CD40	256k	256k	256k	**256k (0)**
Subcutaneous HEL/anti-class II	128k	64k	64k	**85k (21k)**
HEL/anti-class II + 10 μg anti-CD40	64k	64k	64k	**64k (0)**
HEL/anti-class II + 100 μg anti-CD40	64k	64k	128k	**85k (21k)**

Old Mice Group	21 day				45 day			
Intraperitoneal HEL/anti-class II	32k	64k	32k	**42.6k (106k)**	128k	128k	128k	**128k (0)**
HEL/anti-class II + 10 μg anti-CD40	64k	128k	64k	**85k (21k)**	128k	128k	128k	**128k (0)**
HEL/anti-class II + 100 μg anti-CD40	64k	64k	64k	**64k (0)**	nd	256k	512k	**384k (128k)**
Subcutaneous HEL/anti-class II	256k	64k	nd[5]	**160k (96k)**	512k	512k	nd	**512k (0)**
HEL/anti-class II + 10μg anti-CD40	64k	64k	nd	**64k (0)**	256k	256k	nd	**256k (0)**
HEL/anti-class II + 100 μg anti-CD40	128k	64k	128k	**106k (21k)**	256k	512k	512k	**426k (85.3k)**

[1] Female CBA/jNIA mice 1-3 months or 16-19 months of age were inoculated with anti-class II mAb-HEL conjugates either subcutaneously or intraperitoneally with 3.2 ♡g of HEL-anti-class II conjugate with or without anti-CD40 mAb (10 μg or 100 μg).

The construct for this experiment was different from that used for the results presented in Table 1.

[2] Mice were bled on day +21 post-immunization, challenged on day +35 post-immunization and bled again 10 days after the challenge (+45 days). Anti-HEL serum Ig titers were determined for individual mice by ELISA. Prebleed ELISA titers were all <100.

[3] An ELISA titer of <100 is negative. Sample titers were determined by subtracting the sample's absorbance from twice the mean of the blank control wells.

[4] ELISA titers with a "k" after the number should be multiplied by 1000, e.g., 128k is a titer of 128,000, the reciprocal of the serum dilution that was the last positive well.

[5] The mouse died during the experiment and the serum was not collected.

[6] Geometric mean and standard error of mean.

As can be seen from both old and young mice, anti-class II-targeted Hel was extremely immunogenic in this experiment when administered intraperitoneally or subcutaneously (Table 2). Intranasal administration was inductive but not to the degree that the other routes were (data not shown). Similar high titers of anti-HEL serum Ig were noted in the old mice at 21 days post immunization. Following the soluble HEL challenge, the anti-HEL serum Ig titers for the old mice were consistently around 1:256,000. This anti-HEL serum Ig response was remarkably high compared to old mice that respond only marginally to non-targeted HEL/alum (compare Tables 1 and 2). Hence, the inclusion of anti-CD40 mAb in the immunizing inoculum did not result in any enhancement in this experiment.

EXAMPLE 2

Class II-Targeting of DC *In Vitro*, Results in DC That Induce Humoral Immunity

The third HEL experiment was conducted to determine if *in vitro* loading of DC with antigen was similar in efficacy as the *in vivo* priming that was used in the first 2 HEL immunization studies. Fit3-induced DC were enriched from spleens of either old or young mice by B- and T-lymphocyte depletion.

Recovered DCs were treated with soluble HEL, anti-class II-HEL, or anti-class II-HEL plus anti-CD40 mAb as described *supra* in the Materials and Methods section. Given the maturation phenomenon of DCs cultured *in vitro*, in order to ensure optimal loading of antigen, DCs were first allowed to bind the targeting construct at 4°C and then to endocytose the targeted Hel for four hours at 37°C. Anti-CD40 mAb was added to the cultures and the cells were cultured overnight before inoculation. To load DCs with soluble HEL, cells were incubated at 4°C without HEL, and the HEL was added to the culture when the cells were placed at 37°C for overnight incubation. In contrast to the primary responses to HEL that were noted in Table 2, charging DC *in vitro* and using this as an immunogen did not result in anti-HEL serum Ig at day 21 for young or old mice. These results are in Table 3 below.

TABLE 3

Anti-class II targeted antigen can prim dendritic cells *in vitro* for *in vivo* stimulation of humoral responses.

Group - Young Mice	**21 day**[2]			**45 day**			
DCs + soluble HEL[1]	<200[3]	<200	<200	1,600	1,600	800	**1,333**[5] **(266)**
DCs + HEL/anti-class II	<200	<200	<200	12,800	12,800	6,400	**10.4k (2133)**
DCs + HEL/anti-class II + anti-CD40	<200	<200	<200	6,400	6,400	3,200	**533k (1000)**
Group - Old Mice							
DCs + soluble HEL	<200	<200	<200	400	200	<200	**266 (67)**
DCs + HEL/anti-class II	<200	<200	<200	nd[4]	1,600	400	**1,000 (600)**
DCs +HEL/anti-class II + anti-CD40	<200	<200	<200	3,200	3,200	3,200	**3,200 (0)**

[1] Spleens from old (15 month old) or young (2 month old) female CBA/jNIA mice treated with flt3 ligand were collected and B- and T-cells depleted by anti-Thy1 mAb and C' and anti-Ig panning, respectively. Fifty million DC were incubated with anti-class II/HEL (3.2 μg) for 30 minutes at 40°C. Cells were then cultured in 24-well plates at 37°C for four hours before the addition of soluble HEL and/or anti-CD40 mAb (10 μg/ml). After overnight culture, the cells were scraped from the plate, washed twice in PBS, and old or young DC were inoculated subcutaneously into old or young mice.

[2] Mice were bled on day +21 post-immunization, challenged on day +35 post-immunization, and bled again ten days after the challenge (+45). Anti-HEL serum Ig titers were determined for individual mice by ELISA. Prebleed ELISA titers were all <200.

[3] An ELISA titer of <200 is negative. Sample titers were determined by subtracting the sample's absorbance from twice the mean of the blank control wells.

[4] The mouse died during the experiment and no serum was collected.

[5] Geometric mean and standard error of mean.

This is likely due to the lack of B-cell priming resulting from lack of free HEL. However, following the boost with soluble HEL at day +35, the secondary response was improved in the groups of mice that received targeted HEL with or without anti-CD40 mAb. The young mice responded better to the anti-class II-targeted HEL than the old mice, but the old mice in some cases, especially if they received anti-CD40 mAb in addition to the targeted HEL, respond similarly to the young mice. The HEL experiments indicated that *in vitro* anti-class II mAb was indeed an effective targeting substrate. This supports other work that reported antigen targeted to class II molecules *in vivo* was an effective immunogen. Our results indicate that the serologic response in old mice can benefit greatly if antigen is targeted to class II and that DCs loaded *in vitro* can be used to prime T-cells to class II-targeted antigen.

EXAMPLE 3

Surface Expressed Molecules on an APC to Enhance Immune Responses to Targeted Antigen

In order to determine if this antigen targeting effect was restricted to class II on APCs, we examined avidin as an antigen targeted to other APC surface markers. We took advantage of biotinylating mAbs specific for either CD40, CD11c, class II or CD38 to target avidin. This technique was developed by Carayanniotis and Barber and later used to compare the effectiveness of targeting avidin to various surface molecules (Carayanniotis et al, Adjuvant-free IgG responses induced with antigen coupled to antibodies against class II MHC, *Nature*, 327, 59-61, 1987; Skea et al, Studies of the adjuvant-independent antibody response to immuno-targeting: Target structure dependence, isotype distribution, and induction of long-term memory, *J. Immunol.*, 151, 3557-3568, 1993). In the generation of the targeting constructs, avidin was limiting in the conjugation reactions and biotin was constant. Thus, we were able to directly compare the efficacy of targeting the same amount of avidin to specific surface proteins. Analysis of avidin/mAb targeting constructs by SDS-PAGE and Coomasie blue staining indicated that the various specific mAb had the comparable level of avidin associated with them (data not shown).

We tested the efficacy of targeting avidin by CD40 and CD38 specific mAb compared to the serologic response obtained following avidin targeting by anti-class II mAb. These results are in Table 4 below.

TABLE 4

Not all APC surface proteins can be targeted for effective priming for humoral responses.

Group[1]	21 day[2]				45 day			
Avidin/anti-CD40	<100[3]	<100	<100		<100	<100	<100	
Avidin/anti-CD40 + anti-CD40	<100	<100	<100		<100	<100	<100	

Avidin/anti-CD40 + anti-class II	<100	<100	<100		<100	<100	<100	
Avidin/anti-CD38	<100	<100	<100		400	<100	3,200	**1,233 (987)**
Avidin/anti-CD38 + anti-CD40	<100	<100	<100		<100	<100	<100	
Avidin/anti-CD38 + anti-class II	<100	<100	<100		<100	<100	<100	
Avidin/anti-class II	400	200	100	**233[4] (88)**	12,800	3,200	400	**5.47k (3.75k)**
Avidin/anti-class II + anti-CD40	<100	<100	<100		<100	<100	<100	
Avidin/anti-class II + Avidin/anti-CD40	200	400	1,600	**733 (437)**	400	400	6,400	**2.8k (1.83k)**
Avidin (protein) + anti-CD40	<100	<100	<100		<100	<100	<100	
Avidin (Protein) + anti-class II	<100	<100	<100		<100	<100	<100	
Avidin (protein) + anti-class II + anti-CD40	<100	<100	<100		<100	<100	<100	

[1] Female CBA/jNIA mice 10-12 weeks of age were inoculated with avidin conjugated to either anti-class II mAb, anti-CD40 mAb, or anti-CD38 mAb. Mice were immunized on day 0 subcutaneously with 6.5 μg of avidin-mAb conjugate. if unconjugated anti-CD40 mAb or anti-class II mAb was included in the immunizing inoculum it was at a dose of 13 μg or 10 μg, respectively.

[2] Mice were bled on day +21 post-immunization, challenged on day +35 post-immunization, and bled again ten days after the challenge (+45). Anti-avidin serum Ig

responses were determined for the individual mice by ELISA. Prebleed ELISA titers were all <100.

[3] An ELISA titer of <100 is negative. Sample titers were determined by subtracting the sample's absorbence from twice the mean of the blank control wells.

[4] Geometric mean and standard error of mean.

As shown by the results in Table 4, if uncoupled, avidin was used as an antigen in conjunction with a targeting mAb (anti-CD40 or anti-class II), there was no serologic response at 21 days post-immunization, or 10 days afer boosting, indicating that antigen must be directly conjugated to the targeting mAb. Targeting avidin by anti-CD40 mAb did not induce an antibody response to avidin even if non-conjugated anti-CD40 or anti-class II mAb was included in the inoculum. Targeting avidin to CD38 was comparable in effect to targeting by anti-CD40 mAb with the exception that two mice responded after the soluble avidin boost, but none of the anti-CD38 inoculated mice responded if anti-CD40 or anti-class II mAb were also included in the inoculum. As with HEL antigen targeted to class II molecules, avidin conjugated to anti-Class II mAb resulted in a primary response and a secondary response to avidin. Surprisingly, adding anti-CD40 to the initial inoculum was not effective at boosting the response and, in fact, diminished the response unless the anti-CD40 mAb also had avidin attached to it.

EXAMPLE 4

Targeting Potential of anti-CD11c

In another study, we compared the targeting potential of anti-CD11c, another DC surface antigen, to that of anti-CD40 and anti-class II mAbs. In addition to targeting CD11c, we also doubled the immunizing antigen concentration by two-fold, increased the concentration of anti-CD40 mAb in one group, and included a group that was boosted at six days intravenously (IV)

versus subcutaneous boosting at +35 days. The first experimental group examined targeted avidin/mAb alone. Immunization with avidin/anti-CD40 mAb did not induce a primary response but did prime a low secondary response after the +35 day challenge. Increasing the avidin concentration of the class II targeting reagent significantly increased the primary and secondary antibody response (see Tables 3 and 4), and also once again demonstrated the efficacy of targeting antigen to class II molecules. Avidin conjugated to anti-CD11c mAb did not induce a detectable humoral response. Avidin/anti-CD11c mAb plus anti-class II avidin did not induce a significant primary response, but the secondary response was similar to that of class II targeted avidin alone. Anti-CD40 targeted antigen plus anti-class II targeted antigen resulted in a primary response and also a secondary response similar to class II targeting alone. If avidin was targeted by CD11c, class II and CD40 mAbs, there was a marginal response at 21 days in one animal and surprisingly the secondary response was significantly affected in two of three animals compared to using CD11c or CD40 targeting in conjunction with class II targeting.

The same experimental groups were used to test the affect of adding non-targeted, anti-CD40 mAb on the primary and secondary anti-avidin response. These results are in Table 5 (middle panel) below:

TABLE 5

Group[1]								
Subcutaneous								
Avidin/ anti-CD40[1]	<100[3]	<100[3]	<100		1,600	1,600	1,600	1,600 (0)
Avidin/anti-class II	800[2]	6,400	1,600	**2.8k[5] (1.75k)**	32k[4]	64k	32k	**42.6k (10k)**
Avidin/anti-CD11c	<100	<100	<100		<100	<100	<100	

Avidin/anti-CD40 + Avidin/anti-class II	800	400	<100	**433 (202)**	64k	32k	8k	**34.67k (16k)**
Avidin/anti-CD11c + Avidin/anti-class II	<100	200	<100	**133 (33)**	nd	64k	128k	**96k (32k)**
Avidin/-CD11c + Avidin/ 10.2.16 + Avidin/anti-CD40	<100	400	<100	**200 (100)**	4k	128k	4k	**45.3k (41.3k)**
Subcutaneous + anti-CD40								
Avidin/anti-CD40	<100	<100	<100		<100	<100	<100	
Avidin/anti-class II	100	<100	200	**133 (33)**	16k	400	16k	**12k (4k)**
Avidin/anti-CD11c	<100	<100	<100		200	100	100	**133 (33)**
Avidin/anti-CD40 + Avidin/anti-class II	<100	<100	<100		<100	400	400	**300 (100)**
Avidin/anti-CD11c + Avidin/anti-class II	<100	<100	<100		<100	<100	<100	
Avidin/anti-CD11c + Avidin/anti-class II _ Avidin/anti-CD40	<100	<100	<100		400	400	400	**400 (0)**

Subcutaneous + IV challenge + 6d								
Avidin/anti-CD40	<100	<100	<100		16k	16k	16k	**16k (0)**
Avidin/anti-class II	<100	<100	<100		256k	32k	64k	**117k (69.9k)**
Avidin/anti-CD11c	6,400	800	1,600	**2.8k (1.75k)**	16k	32k	16k	**21.3k (5.3k)**
Avidin/anti-CD40 + Avidin/anti-class II	<100	<100	<100		64k	16k	64k	**48k (16k)**
Avidin/anti-CD11c + Avidin/anti-class II	1,600	400	400	**800 (400)**	64k	64k	128k	**85.3k (21.3k)**
Avidin/anti-CD11c + Avidin/anti-class II + Avidin/anti-CD40	100	800	800	**567 (233)**	32k	32k	32k	**32k (0)**

[1] Female CBA/jNIA mice 8-10 weeks of age were inoculated with avidin conjugated to either anti-class II mAb, anti-CD40 mAb or anti-CD11c mAb. Mice were immunized on day 0 subcutaneously with 12.5 μg of avidin-mAb conjugate. Titer shown are for individual mice. Unconjugated anti-CD40 mAb (30 μg) was inoculated at the time of the avidin/mAb constructs. Mice challenged at +6 days post-immunization were given 25 μg of avidin intravenously via the tail vein.

[2] Mice were bled on day +21, post-immunization, and either challenged subcutaneously on day +35 post-immunization and bled again ten days after the challenge (+45) or challenged intravenously on day +6 post-immunization. Anti-avidin serum Ig was determined for individual mice by ELISA. Prebleed ELISA titers were all <100.

[3] An ELISA titer of <100 is negative. Sample titers were determined by subtracting the sample's absorbance from twice the mean of the blank control wells.

[4] ELISA titers with a k after the number should be multiplied by 1000, e.g., 32k is a titer of 32,000, the reciprocal of the serum dilution that was the last positive well.

[5] Geometric mean and standard error of mean.

The marginal primary responses that were reported above with avidin/anti-class II was either eliminated or significantly reduced by the inclusion of anti-CD40 mAb at the time of immunization. The trend was also seen in the secondary antibody response for mice immunized with combinations of targeting reagents. For the secondary response, consistent with the efficacy of targeting antigen to class II, the avidin/anti-class II induced response in the presence of anti-CD40 mAb was only reduced in one mouse.

The third parameter of this study examined the affect of IV challenge at +6 days post immunization instead of +35 days. The rationale for this change was to provide soluble antigen for B-cells at the time when germinal center formation would be at its height from the primary immunization. These experiments revealed an interesting change in the immune response of mice immunized with avidin/mAb. If the challenge with soluble antigen was at six days post immunization, then the 21 day primary response was now only seen in mice that were immunized with avidin/anti-CD11c either with or without other targeting reagents. The secondary responses were similar to those seen with the same groups that were challenged at +35 days, with the exception of avidin/anti-CD40 and avidin/CD11c which were greatly improved. Challenge at +6 days increased the secondary response or normalized the response for the animals within the combination therapy group (avidin/CD11c, avidin/CD40, and avidin/class II).

CONCLUSIONS

Targeted Antigen Can Enhance Serologic Responses

Our results indicate that anti-class II mAb are an effective method to induce humoral responses in aged mice. Our work was based on previous studies that demonstrated anti-class II mAb can be used in young mice to target antigen

more effectively than non-targeted antigen (Snider et al, *J. Exp. Med.*, 171, 1957-1963 (1990); Estrada et al, *Vaccine*, 13:901-907 (1995); Snider et al, *Immunol.*, 90:434-439 (1997).) Significantly, our results demonstrated that anti-class II mAb can be used to enhance serologic responses of aged mice to protein antigens that are marginally immunogenic when delivered in alum. Targeting antigen through class II molecules works both *in vivo and in vitro*, although the *in vitro* targeting was not as effective as *in vivo* targeting. The reason for the difference is not known, but may relate to the premature maturation of DC in culture which would reduce their ability to endocytose antigen as efficiently as DC targeted with antigen *in vivo*. A more likely explanation is inefficient B-cell priming. If the DC are targeted *in vitro*, less free antigen is available to prime B-cells for antibody production. The mechanism whereby class II-targeted antigen enhances immune responses has not been completely elucidated, however, one study did examine the distribution of radiolabeled class II-targeted antigen into the lymph nodes following inoculation in the foot pad. The intense DC labeling that was reported shortly after inoculation suggests that the label as not transported to the lymph node by migrating DC but rather a substantial amount directly targets APC in draining lymph node. Anti-class II targeting constructs were found to label interdigitating cells (DCs in the T-cell area) in the paracortex and were detected up to sixteen days following injection. Non-targeting antibody constructs were taken up by macrophages and catabolized very quickly. While it is clear that anti-class II mAb can target antigen to APC in the T-cell areas of the lymph node, the affect on the APC is not known.

<u>The APC Molecule Targeted and the Other Concurrent Receptor Events are Important for Enhancing the Primary Humoral Response</u>

If the mechanism of enhanced response to a targeted antigen is solely attributed to increased antigen retained by DC in the lymph node and thus greater

T-cell priming, then other DC surface molecules should also be able to provide a similar affect if targeted. In a previous study, it was reported that class I and FcγRII antibodies used to target antigen can enhance serologic responses to targeted antigen, but anti-IgD mAb to target antigen to B-cells cannot (Snider et al, *J. Exp. Med.*, 171:1957-1963 (1990).) This result fits well with the notion that DCs are the main APC that stimulates a primary response and further suggests that multiple surface molecules could serve as targets. To test this, we examined the efficacy of targeting different APC surface molecules for subsequent induction of humoral responses. We used avidin as an antigen conjugated to anti-class II mAb (DC, Mø, B-cells), anti-CD40 mAb (DC, Mø, B-cells), anti-CD11c mAb (DC and myeloid cells), or anti-CD38 mAb (B-cells). Anti-class II mAb, anti-CD40 mAb and anti-CD11c mAb would target similar cell populations in lymph nodes. The use of CD11c mAb would not target B-cells, while anti-CD38 would not target DC or Mø (Oliver et al, *J. Immunol.*, 158:1108-1115 (1997).) Consistent with the anti-IgD targeting of antigen, using anti-CD38 to target B-cells does not enhance serologic responses.

In support of only a specific set of APC surface molecules being useful as a targeting substrate, targeting avidin to CD40 or CD11c expressed on a DC or Mø does not ensure an enhanced serologic response. Class II-targeted antigen is superior compared to CD40 or CD11c targeting. The reasons for this difference is not known but it is important to emphasize that class II (Wade et al, *Immun. Today*, 14:539-546 (1998), CD40 (van Kooten et al, *Cur. Opin. Immunol.*. 9:330-337 (1997), and CD11c (De La Salle et al, *Adv. Exp. Med. Biol.*, 417:345-351 (1997); Medvedev et al, *J. Immunol.*, 160:4535-4542 (1998), all act as signal transduction molecules on APC. Further, all have been reported to be involved in antigen presentation. It is unclear whether the generation of different

signals by CD40 or CD11c ligation compared to class II ligation may affect the efficacy of priming.

Also, it is hypothesized that the enhancing effect of targeted antigen to class II may be due to the qualitative or quantitative access of class II/antigen complexes to the endocytic following endocytosis. Still alternatively, the explanation for the differences in antigen targeting may be attributable to a difference in level of expression or APC subset distribution of the targeted molecule. Future studies should determine what parameters of the targeted surface molecule are significant for enhanced serologic responses to targeted antigen. If the mechanism of affect can be determined, protocols that optimize the adjuvant affect of targeted antigens can be used to effectively immunize aged individuals. This is particularly attractive strategy for antigens that need to induce a protective Th1, cell-mediated immunity.

Consequences of CD40 Ligation During Immunization With Targeted Antigen

CD40 ligation has a dramatic affect on DC biology and maturation. It can increase IL-12 secretion and up-regulate costimulatory molecules (Scheidegger et al, *J. of Exp. Med.*, 184:747-752 (1996).) Mature DC have reduced internalization of Fc and mannose receptors and have increased expression and half-life of class II/peptide complexes (Cella et al, *Nature*, 388:782-787 (1997).) This may explain why ligation of CD40 during delivery of targeted immunogens can have an effect on subsequent immune responses. We demonstrated an increase in the serologic response to HEL in mice treated with anti-CD40 mAb *in vivo*, and also for mice immunized with antigen pulsed DC treated with anti-CD40 mAb *in vitro*. However, in another antigen system, targeted avidin, CD40 ligation was in general suppressive to subsequent humoral responses. Clearly, if we are to consistently take advantage of the adjuvant or immunosuppressant properties of CD40 ligation, the dynamics of antigen targeting so as to interpose

CD40 at the optimal moment or location will need to be determined for the particular antigen system. In this regard, effective manipulation of APC surface molecules *in vivo* may not be possible. Alternatively, isolation of DC and controlled signaling during antigen delivery *in vitro* could be used to improve the immunogenicity of DC pulsed with tumor antigens.

While the invention has been described with respect to certain specific embodiments, it will be appreciated that many modifications and changes thereof may be made by those skilled in the art without departing from the spirit of the invention. It is intended, therefore, by the appended claims to cover all modifications and changes that fall within the true spirit and scope of the invention.

WHAT IS CLAIMED IS:

1. A method for enhancing or suppressing at least the humoral immune response or CD4 ThI immune response to a target antigen comprising administering the following:

(i) a conjugate comprising a selected antigen, which is directly or indirectly attached to an antibody that specifically binds to a molecule which is expressed by an antigen-presenting cell (APC); and

(ii) an anti-CD40 antibody, wherein the antigen-antibody conjugate of (i) and the anti-CD40 antibody of (ii) are administered simultaneously or substantially contemporaneously.

2. The method of claim 1 wherein the antibody attached to said antigen is selected from the group consisting of an anti-MHC class II antibody, an anti-MHC class I antibody, an anti-CD11c antibody, an anti-dendritic cell antigen antibody, an anti-follicular cell antigen antibody, and an anti-Fc molecule antibody.

3. The method of Claim 1, wherein said antibody specifically binds a human class II MHC molecule or CD11c.

4. The method of Claim 1, which is effected *in vitro* by contacting antigen-presenting cells to said conjugate (i) and anti-CD40 antibody (ii).

5. The method of Claim 1, which is effected *in vivo*.

6. The method of Claim 5, wherein said method is effected in an aged or immuno-compromised individual.

7. The method of Claim 6, wherein the treated individual is a human subject fifty years or older.

8. The method of Claim 6, which is used for the treatment of viral infection, bacterial infection or cancer.

9. The method of Claim 1, wherein said antigen is expressed by a moiety selected from the group consisting of a tumor or cancer cell, a virus, a pathogen, a bacterium, a fungi, and a toxin.

10. The method according to Claim 9, wherein said cancer or tumor cell is selected from the group consisting of prostate, breast, ovarian, lung, head and neck, uterine, and leukemia.

11. The method according to Claim 9, wherein said virus is selected from the group consisting of a papillomavirus, RSV, herpes virus, in influenza virus, a hepatitis virus, a polio virus, and HIV virus.

12. The method according to Claim 1, wherein the antigen-antibody conjugate of (i) and the anti-CD40 antibody are administered together.

13. The method according to Claim 1, wherein the antigen is directly attached to said antibody.

14. The method according to Claim 13, wherein said direct attachment comprises covalent attachment of the antigen and the antibody.

15. The method according to Claim 1, wherein the administered antigen-antibody conjugate of (i) and the anti-CD40 antibody are contained in the same composition.

16. The method of Claim 1 which is used to induce a protective Th1 cell-mediated immune response against a bacterial disease or protozoan disease.

17. The method of Claim 1 which is used to treat leishmanin????, Listerine?????, leprosy, or tuberculosis infection.

18. A composition adopted for enhancing or suppressing an immune response to a selected antigen which comprises at least the following:

(i) a selected antigen which is directly or indirectly attached to an antibody that specifically binds to an antigen expressed by an antigen-presenting

cell (APC) selected from the group consisting of dendritic cells, B-cells, macrophages and follicular dendritic cells; and

(ii) an anti-CD40 antibody, wherein the antigen-antibody conjugate of (i) and the anti-CD40 antibody of (ii) are contained in amounts effective to enhance the humoral immune response to the antigen, relative to the antibody production obtained when said same antigen is administered at the same amounts, in the absence of the anti-CD40 antibody, and in unconjugated form.

19. The composition according to Claim 18, wherein the antibody attached to said antigen is selected from the group consisting of an anti-MHC class II antibody, an anti-MHC class I antibody, an anti-B7 antibody, an anti-dendritic cell antigen antibody, an anti-follicular cell antigen antibody, and an anti-Fc molecule antibody.

20. The composition according to Claim 19, wherein said antibody is an anti-MHC class II antibody.

21. The composition according to Claim 19, wherein said antigen is expressed by a moiety selected from the group consisting of a tumor or cancer cell, a virus, a pathogen, a bacterium and a fungus.

22. The composition according to Claim 19, wherein said virus is selected from the group consisting of a papillomavirus, a herpes virus, an influenza virus RSV, a hepatitis virus, a polio virus, and an HIV virus.

23. The composition according to Claim 18, wherein said tumor or cancer cell is selected from the group consisting of prostate, breast, ovarian, lung, head and neck, uterine, leukemia, skin, bladder, or melanoma.

24. The composition according to Claim 18, which further comprises an adjuvant.

25. The composition according to Claim 24, wherein said adjuvant is selected from the group consisting of Alum, saponin, and Freund's complete adjuvant.

26. The composition according to Claim 18, wherein said anti-CD40 antibody is selected from the group consisting of a humanized antibody, a chimeric antibody, a human monoclonal antibody, or a fragment thereof that specifically binds CD40.

27. The composition according to Claim 18, wherein said antibody that specifically binds to an antigen expressed by an antigen presenting cell (APC) is selected from the group consisting of a human monoclonal antibody, a chimeric antibody, a humanized monoclonal antibody, and a fragment thereof that specifically binds to said antigen expressed by an antigen presenting cell.

28. The composition of Claim 18, which affects at least one of CD4, ThI activation, IL12, and γ interferon expression.

29. A kit adopted for enhancing at least the humoral immune response or to a particular DTH type T-cell response antigen upon administration to a host in need of such treatment which comprises at least the following:

(i) a selected antigen which is directly or indirectly attached to an antibody that specifically binds to an antigen expressed by an antigen-presenting cell (APC); and

(ii) an anti-CD40 antibody, wherein such moieties may be packaged separately or in combination.

30. The kit according to Claim 27???, which further comprises at least one additional moiety selected from the group consisting of a stabilizer, an adjuvant, a surfactant, a fungicidal agent, a bactericidal agent, a pharmaceutically acceptable carrier or excipient, and an immunostimulating peptide.

31. A method for enhancing the humoral or DTH ThI type immune response to a target antigen in an individual that is immuno-compromised because of age, disease, or genetic defect, comprising administering to said subject (i) a conjugate comprising said target antigen attached directly or indirectly to an antibody specific to an anti-MHC class II molecule, and (ii) optionally an antibody specific to CD40.

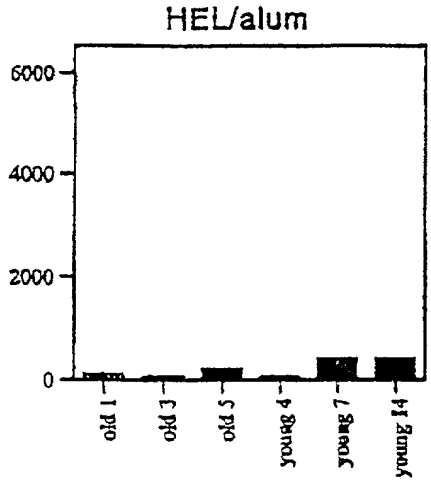

FIG 1-A

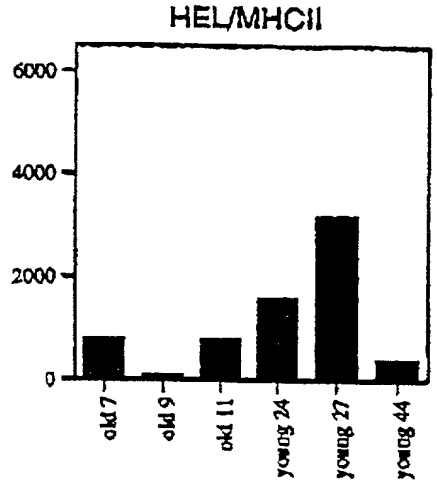

FIG 1-B

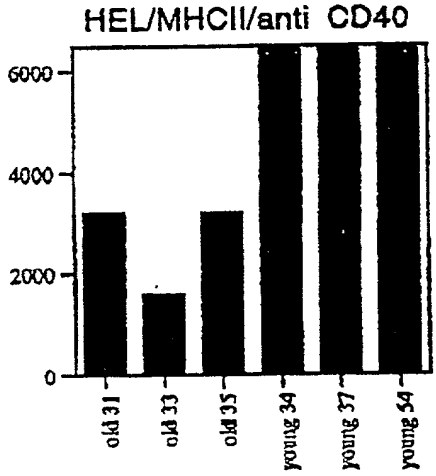

FIG 1-C

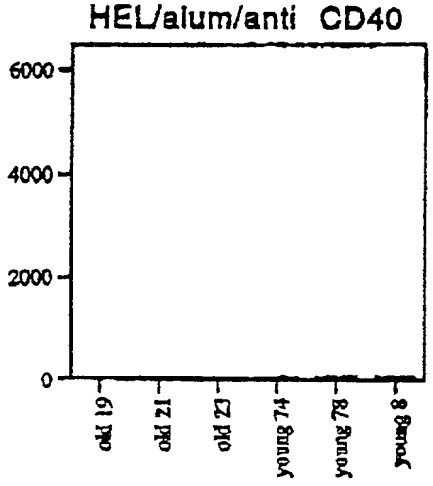

FIG 2-A

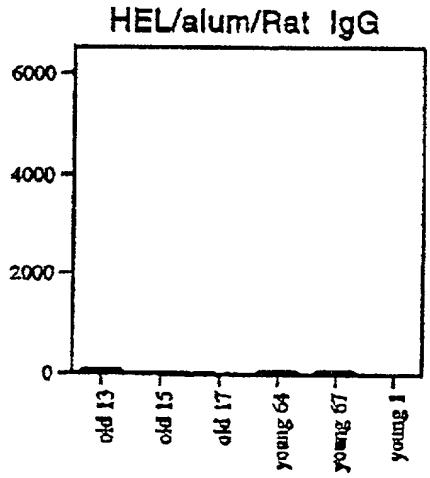

FIG 2-B

FOR UTILITY/DESIGN CIP/PCT NATIONAL/PLANT ORIGINAL/SUBSTITUTE/SUPPLEMENTAL DECLARATIONS

RULE 63 (37 C.F.R. 1.63) DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

PW FORM

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name, and I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the **INVENTION ENTITLED**

METHODS AND COMPOSITIONS FOR MODULATING ANTIGEN-SPECIFIC IMMUNOLOGICAL (HUMORAL) RESPONSES BY TARGETING SUCH ANTIGEN TO APCs IN CONJUNCTION WITH ANTI-CD40 LIGAND ADMINISTRATION

the specification of which (CHECK applicable BOX(ES))

X A. ☐ is attached hereto.

BOX(ES) → B. ☐ was filed on _____ as U.S. Application No. _____ /

→ → C. ☒ was filed as PCT International Application No. PCT/ US99/12825 on June 25, 1999

and (if applicable to U.S. or PCT application) was amended on _____

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose all information known to me to be material to patentability as defined in 37 C F R 1 56 Except as noted below, I hereby claim foreign priority benefits under 35 U S C 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT International Application which designated at least one other country than the United States, listed below and have also identified below any foreign application for patent or inventor's certificate, or PCT International Application, filed by me or my assignee disclosing the subject matter claimed in this application and having a filing date (1) before that of the application on which priority is claimed, or (2) if no priority claimed, before the filing date of this application

PRIOR FOREIGN APPLICATION(S) Number	Country	Day/MONTH/Year Filed	Date first Laid-open or Published	Date Patented or Granted	Priority NOT Claimed

If more prior foreign applications, X box *at bottom* and continue on attached page.

Except as noted below, I hereby claim domestic priority benefit under 35 U S C 119(e) or 120 and/or 365(c) of the indicated United States applications listed below and PCT international applications listed above or below and, if this is a continuation-in-part (CIP) application, insofar as the subject matter disclosed and claimed in this application is in addition to that disclosed in such prior applications, I acknowledge the duty to disclose all information known to me to be material to patentability as defined in 37 C.F.R. 1.56 which became available between the filing date of each such prior application and the national or PCT international filing date of this application:

PRIOR U.S. PROVISIONAL, NONPROVISIONAL AND/OR PCT APPLICATION(S) Application No. (series code/serial no.)	Day/MONTH/Year Filed	Status pending, abandoned, patented	Priority NOT Claimed
60/090,849	26 June 1998	pending	

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

And I hereby appoint Pillsbury Winthrop LLP, Intellectual Property Group, telephone number (202) 861-3000 (to whom all communications are to be directed), and persons of that firm who are associated with USPTO Customer No 909 (see below label) individually and collectively my attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and with the resulting patent, and I hereby authorize them to delete from that Customer No. names of persons no longer with their firm, to add new persons of their Firm to that Customer No., and to act and rely on instructions from and communicate directly with the person/assignee/attorney/firm/ organization who/which first sends/sent this case to them and by whom/which I hereby declare that I have consented after full disclosure to be represented unless/until I instruct the above Firm and/or an attorney of that Firm in writing to the contrary

USE ONLY FOR PILLSBURY WINTHROP

William F Wade

00909

William F Wade

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Mailing Address	Dartmouth Medical School-Dept of Microbiology, 603 W. Borwell Bldg, One Medical Center Drive		
(include Zip Code)	Lebannon, New Hampshire 03756		

☐ FOR ADDITIONAL INVENTORS see attached page.

☐ See additional foreign priorities on attached page (incorporated herein by reference).

Atty. Dkt. No. P0276517 (M#)

CIP/PCT NATIONAL/PLANT ORIGINAL/SUBSTITUTE/SUPPLEMENTAL DECLARATIONS

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

FORM

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name, and I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the **INVENTION ENTITLED**

METHODS AND COMPOSITIONS FOR MODULATING ANTIGEN-SPECIFIC IMMUNOLOGICAL (HUMORAL) RESPONSES BY TARGETING SUCH ANTIGEN TO APCs IN CONJUNCTION WITH ANTI-CD40 LIGAND ADMINISTRATION

the specification of which (CHECK applicable BOX(ES))

X A. ☐ is attached hereto.

BOX(ES) → B. ☒ was filed on December 22, 2000 as U.S. Application No. 09/720,078

→ → C. ☒ was filed as PCT International Application No. PCT/ US99/12825 on June 25, 1999

and (if applicable to U.S. or PCT application) was amended on

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose all information known to me to be material to patentability as defined in 37 C.F.R. 1.56 Except as noted below, I hereby claim foreign priority benefits under 35 U.S C. 119(a)-(d) or 365(b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT International Application which designated at least one other country than the United States, listed below and have also identified below any foreign application for patent or inventor's certificate, or PCT International Application, filed by me or my assignee disclosing the subject matter claimed in this application and having a filing date (1) before that of the application on which priority is claimed, or (2) if no priority claimed, before the filing date of this application:

PRIOR FOREIGN APPLICATION(S) Number	Country	Day/MONTH/Year Filed	Date first Laid-open or Published	Date Patented or Granted	Priority NOT Claimed

If more prior foreign applications, X box *at bottom* and continue on attached page.

Except as noted below, I hereby claim domestic priority benefit under 35 U S C. 119(e) or 120 and/or 365(c) of the indicated United States applications listed below and PCT international applications listed above or below and, if this is a continuation-in-part (CIP) application, insofar as the subject matter disclosed and claimed in this application is in addition to that disclosed in such prior applications, I acknowledge the duty to disclose all information known to me to be material to patentability as defined in 37 C.F.R. 1 56 which became available between the filing date of each such prior application and the national or PCT international filing date of this application:

PRIOR U.S. PROVISIONAL, NONPROVISIONAL AND/OR PCT APPLICATION(S) Application No. (series code/serial no.)	Day/MONTH/Year Filed	Status pending, abandoned, patented	Priority NOT Claimed
60/090,849	26 June 1998	pending	

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

And I hereby appoint Pillsbury Winthrop LLP, Intellectual Property Group, telephone number (703) 905-2000 (to whom all communications are to be directed), and persons of that firm who are associated with USPTO Customer No. 909 (see below label) individually and collectively my attorneys to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith and with the resulting patent, and I hereby authorize them to delete from that Customer No. names of persons no longer with their firm, to add new persons of their Firm to that Customer No , and to act and rely on instructions from and communicate directly with the person/assignee/attorney/firm/ organization who/which first sends/sent this case to them and by whom/which I hereby declare that I have consented after full disclosure to be represented unless/until I instruct the above Firm and/or an attorney of that Firm in writing to the contrary

USE ONLY FOR PILLSBURY WINTHROP

00909

00909

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(2) **INVENTOR'S SIGNATURE:** **Date:**

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	First	Middle Initial	Family Name
Residence			
	City	State/Foreign Country	Country of Citizenship
Mailing Address			
(include Zip Code)			

☐ FOR ADDITIONAL INVENTORS see attached page.

☐ See additional foreign priorities on attached page (incorporated herein by reference).

Atty. Dkt. No. P276517 (M#)